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Award Number: W81XWH-10-1-0105

TITLE: Targeting PCNA Phosphorylation in Breast Cancer

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REPORT DATE: April 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
April 2011	Annual	15 March 2010 – 14 March 2011

4. TITLE AND SUBTITLE

Targeting PCNA Phosphorylation in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0105

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Purdue University
West Lafayette, IN 47907

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

PCNA, an essential protein in the DNA synthesome, was shown to be phosphorylated at Y211 by a nuclear form of EGFR. A consequence of this alteration has been highly correlated with a reduced survival rate of breast cancer patients. However, no basic or clinical studies have addressed if these inhibitors down-regulate the nuclear function of this protein. Inhibitors of EGFR have exciting potential in other cancer diseases but have failed to show clinical efficacy as a mono-therapy treatment option for breast cancer patients. In addition, therapy resistance in breast cancer patients who initially responded to the inhibitor of ErbB2 receptor tyrosine kinase Lapatinib is now known. Together, these observations call for more specific markers to stage breast cancer patients for effective use of this class of molecular therapeutics. A focused approach to enhance tyrosine kinase inhibitors by intracellular targeting is novel approach under investigation. A main focus of the proposal is to assess if a specific post-translational modification could be an associated specificity marker for malignant tumors.

15. SUBJECT TERMS
PCNA, surface enhanced Raman, nuclear localization, kinase pathway inhibitors, DNA replication, DNA repair

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU		

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Introduction*:* Receptor tyrosine kinases are pivotal players in the control of cellular progression and differentiation. These enzymes predominantly reside at the plasma membrane and initiate signaling cascades as a result of an external stimuli. The expression and dysregulation of ErbB, Eph, and VEGF receptor tyrosine kinases have shown to be present in breast cancer. Recent evidence implicates receptor tyrosine kinases also playing vital roles in downstream processes including tyrosine phosphorylation of non-signaling proteins. Specifically, PCNA, an essential protein in the DNA synthesome, was shown to be phosphorylated at Y211 by a nuclear form of EGFR. A consequence of this alteration has been highly correlated with a reduced survival rate of breast cancer patients. However, no basic or clinical studies have addressed if these inhibitors down-regulate the nuclear function of this protein. Inhibitors of EGFR have exciting potential in other cancer diseases but have failed to show clinical efficacy as a mono-therapy treatment option for breast cancer patients. In addition, therapy resistance in breast cancer patients who initially responded to the inhibitor of ErbB2 receptor tyrosine kinase lapatinib is now known. Together, these observations call for more specific markers to stage breast cancer patients for effective use of this class of molecular therapeutics.

Basic Hypothesis: Tyrosine phosphorylation of PCNA is a cancer-specific regulator of DNA repair. This project will test the role of receptor tyrosine kinases in PCNA phosphorylation and assess the pharmacologic targeting of nuclear tyrosine kinases.

Key Experimental Methodologies and Approach: To evaluate if candidate tyrosine kinases represented in the nucleus of breast tumor cells accept as a substrate the unique sequence in PCNA, a series of *in vitro* biochemical analyses with tyrosine kinases are being conducted. A small library of synthetic peptides based upon the unique sequence incorporating Y211 in PCNA is being used to evaluate the relative selectivity of phosphorylation and/or kinase inhibition. PCNA mimics are prepared using solid phase methods based upon sequence 207-215 (FALRYLNFF) which is unique in the human genome. The relative ability of the peptides to be phosphorylated by nuclear kinases is being assessed using capillary electrophoresis. The optimal peptide(s) for interactions with receptor tyrosine kinases including EGFR will be selected for subsequent structure-based inhibitor design. Additionally, these peptide tools will be used to evaluate nuclear tyrosine kinase(s) responsible for phosphorylation of Y211-PCNA in breast tumor cell models. Therapeutic tyrosine kinase inhibitors are being evaluated for their relative capacity to inhibit phosphorylation of Y211 in PCNA and the peptide substrate mimics developed herein. Nuclear delivery of known experimental therapeutics will be explored to assess if nuclear tyrosine kinase inhibition is a useful pharmacological endpoint in breast cancer models. Using synthetic modifications of existing experimental therapeutics with known receptor tyrosine kinase inhibitory activities, these drugs will be targeted to the nucleus of breast cancer tumor cell models. Finally, ultra-sensitive detection technologies with high capacity proteomic separation are being evaluated for use in measurement of pharmacodynamic markers that could be used for translation to the clinic.

Progress on Original Statement of Work: The start of the project on all aims was delayed 6 months due to change of personnel and recruitment of new associates to fill out a full team. Therefore, several specific tasks were not initiated in the reporting period and there was no cost to the project. The overall progress during the specific reporting period represents only a portion of the proposed total effort. The subsequent reporting periods will reflect an accelerated progress with increased time commitment from more research associates which are all now in place.

Aim 1a. To evaluate candidate tyrosine kinases potentially represented in the nucleus of tumor cells for their capacity to selectively target a unique sequence in PCNA including Y211.

The tasks associated with this subaim were delayed due to change and availability of personnel and a major technical problem with instrumentation. In particular, the capillary electrophoresis system required significant upgrades and methods development before application to this project. The exception to this is Task 2 which has been now accomplished multiple times at this point on a synthetic scale that reduces to practice the peptide series originally proposed to be available in reagent quantities.

Task 1. Recombinant expression and purification of different tyrosine kinases.

This task has been pursued and technical challenges have been identified requiring significant optimization for heterologus expression of the receptor tyrosine kinase domains. Alternative commercial sources were used to initiate studies of the substrates based upon PCNA.

Task 2. Synthesis of the unique peptide sequence modeled after PCNA (containing Y211)

A series of peptides based upon the Y211 phosphorylation site in PCNA have been prepared for study. See Appendix 1

Task 3. Development of an endpoint assay to measure pY211/Y211 levels using capillary electrophoresis

Task 4. Determine basic turnover kinetics using capillary electrophoresis

Aim 1b. To evaluate the nuclear kinase activities responsible for phosphorylation of PCNA at Y211 in tumor cell models.

The progress to date on this subaim is focused on methods development and implementation. Task 1 has been accomplished and is ongoing process. Methods for subcellular fractionation of tumor cells are implemented and validated in Appendix 4. Efforts on Tasks 3 and 4 have been initiated now with the upgrade of instrumentation.

Task 1. Growth and maintenance of breast cancer cell lines with and without inhibitor added

See Appendix 2

Task 2. Prepare nuclear extracts of breast cancer cell lines using sucrose gradient

See Appendix 4

Task 3. Perform Western Blot analysis, investigating expression of select nuclear tyrosine kinases

Task 4. Determine the ability of the unique peptide sequence to be phosphorylated in nuclear extracts of breast cancer cells with and without inhibitors

Aim 2a. Using a structure-based approach, known experimental therapeutics will be targeted to the nucleus in breast cancer tumor cell models by synthetic incorporation of nuclear-delivery features.

The primary progress on this subaim has been focused on sourcing of chemical agents that represent a range of protein kinase inhibitors. A key kinase inhibitor analog has been made that is suitable for subsequent chemical modification for inclusion of a nuclear localization sequence. Efforts have been initiated toward Tasks 2-6.

Task 1. Acquire necessary tyrosine kinase inhibitors

See Appendix 3

A collection of 14 inhibitors have been acquired or synthesized. One of these compounds is a novel analog related to the tyrosine kinase inhibitor gefitinib. This compound serves as the precursor for convergence with the peptide and peptoid nuclear localization targeting sequences.

Task 2. Synthesize peptoid nuclear localization sequence

Task 3. Validate the nuclear localization sequence does target the nucleus of the breast cancer cells

Task 4. Couple peptoid nuclear localization sequence to the tyrosine kinase inhibitor

Task 5. Perform colocalization studies using fluroescence microscopy to validate localization of inhibitor to the nucleus

Task 6. Perform cell growth inhibition assays using breast cancer cell lines and the nuclear-localized tyrosine kinase inhibitors

Aim 2b. Using novel, ultra-sensitive, quantitative Raman-based detection tools, the capacity of nuclear-targeted tyrosine kinase inhibitors to alter the PCNA pY211/Y211 content in breast tumor cell models will be pursued.

The progress on this aim has been entirely focused on methods development and optimization for separation of PCNA protein isoforms, quantification using reverse immunological assays, and comparison with standard ELISA assays. A new labeling reagent process for production and scale up has been developed that is now more efficient and reproducible. For Tasks 1-4, the preliminary data and methods have been developed and are being validated.

Task 1. Further develop FFE isolation and SERRS detection platforms to quantitative assessment of pY211/Y211 levels of PCNA

See Appendix 4

Task 2. Prepare nuclear extracts of breast cancer cell lines using sucrose gradient

See Appendix 4

Task 3. Separate nucelar protein extracts using FFE

See Appendix 4

Task 4. Perform dot blot analysis of the nuclear protein FFE fractions

See Appendix 4

Task 5. Analyze pY211/Y211 levels of PCNA using SERRS detection

KEY RESEARCH ACCOMPLISHMENTS:

Established key technical components required for the overall project.

Established key personnel needed and recruited to the project.

Feasibility of antibody containing equal levels of isotopic dye variants has been established.

Proof of concept application of Raman-based detection combined with reverse immunological assays for quantification of cancer specific PCNA-isoforms has been accomplished.

A biologically active receptor tyrosine kinase inhibito with suitable functionality for use in click reaction to conjugation dyes and nuclear targeting sequences has been successfully produced.

REPORTABLE OUTCOMES:

Era of Hope 2011, August 2-5, 2011 presentation of poster entitled: “Targeting PCNA Phosphorylation in Breast Cancer” V. Jo Davisson, Anthony Pedley, Qingshou Chen, Matt Bartolowits.

REFERENCES: N/A

Appendix 1: Peptide Synthesis

Synthetic peptides will be used in the confirmation of whether or not nuclear tyrosine kinases accept a PCNA-specific sequence that includes the amino acid residue tyrosine 211 (Y211). Relative amounts of phosphorylation of the difference peptide sequences by various tyrosine kinases will be quantified using capillary electrophoresis (equipped with a photodiode array) and this information will be used to argue that nuclear tyrosine kinases directly interact with and phosphorylate PCNA.

Three peptides derived from the twelve amino acid residues surrounding PCNA Y211 have been synthesized as well as a randomized sequence to serve as a control. The randomized sequence, henceforth referred to as "scrambled," contains the same residues as the PCNA derived sequences, but in a randomized order.[1] The peptides, written N-terminal to C-terminal from left to right, are as follows:

PCNA Y211: TFALRYLNFFTK	anticipated m/z 1520.8260, observed m/z 1520.7942
PCNA Y211F: TFALRFLNFFTK	anticipated m/z 1504.8311, observed m/z 1504.7515
PCNA pY211: TFALRpYLNFFTK	anticipated m/z 1600.7923, observed m/z 1599.8541
PCNA scrambled: FLFTNKLFRTAF	anticipated m/z 1504.8311, observed m/z 1503.8320

The PCNA Y211F peptide is used as a negative control—tyrosine being replaced with phenylalanine should not allow for phosphorylation. PCNA pY211 is used as a positive control as the tyrosine residue remains phosphorylated.

Methods

The peptides used in this study have been synthesized by two methods: manual solid phase synthesis and automated synthesis using an Invatas peptide synthesizer. Identity and amino acid order are confirmed using MALDI and TOF-TOF mass spectrometry.

Solid Phase Peptide Synthesis

0.100 mmol of Rink Amide Tentagel Resin (substitution value: 0.71 mmol eq/g resin) is weighed and transferred to a peptide reaction vessel. 5mL of dimethylformamide (DMF) is added to the resin and the resin is allowed to swell for 30 minutes. The DMF is removed, and 3mL of a 20% piperidine in DMF solution is added to the reaction vessel containing the resin; the resin is allowed to incubate at room temperature for 30 minutes with shaking in order to deprotect the resin. After the 30 minute incubation, the resin is washed six times with DMF and then three times with dichloromethane (DCM). Deprotection is confirmed via a Ninhydrin test (Kaiser's test; described below). A solution of 2.1 ml 0.45M O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) in DMF, 435µl 2M diisopropylethylamine (DIEA), and 1 mmol (10 equivalents) of the respective Fmoc-protected amino acid are added to the resin, and the resin is incubated at room temperature with shaking for one hour. After one hour, the resin is washed six times with DMF and three times with DCM. Residue coupling is confirmed via a Ninhydrin test. The deprotection and coupling steps are repeated until the entire peptide has been synthesized. After the final amino acid residue has been deprotected and the resin washed as before, a solution of TFA/H_2O/TIS (95:2.5:2.5) is added to the resin with incubation at room temperature for four hours. At that time, the resin is filtered from the supernatant and the peptide is precipitated into diethyl ether. The peptide is then stored at -20°C.

In preparation for mass spec analysis and peptide purification, the diethyl ether is evaporated and the remaining residue is dissolved in acetonitrile/water (1:1).

Ninhydrin (Kaiser's) Test

Solutions used:

- 5g ninhydrin in 100ml ethanol

- 80g phenol in 20ml ethanol
- .02 mM potassium cyanide in pyridine

To a small amount of washed resin is added three drops of each of the three solutions used in the test. The resin is placed at 100°C for five minutes—at that time, a dark blue solution indicates the presence of a primary amine (deprotected residue); otherwise, the solution remains yellow.

Peptide Purification

The peptides are purified on a reverse phase semi-prep HPLC column using a gradient of acetonitrile with 0.1% TFA / water with 0.1% TFA from (5:95) to (100:0) over the course of 30 minutes. The flow-through is collected and lyophilized to give the purified peptide.

References

1. Zhao, H., et al. Targeting Tyrosine Phosphorylation of PCNA Inhibits Prostate Cancer Growth. *Mol. Cancer Ther.* (2011), 10, 29-36.

Appendix 2. Breast Tumor Cell Line Panel

The following cell lines have been acquired, in cultivation, and are currently available for testing.

cell line	HER2	ER	PR	p53	BRCA1
SKBR3	+	-	-	mutant	WT
MDA-MB-231	-	-	-	mutant	WT
MDA-MB-468	-	-	-	mutant	WT
MCF7	-	+	+	WT	WT
BT474	+	+	+	WT	WT
UACC812	+	+	-	WT	WT
ZR75B	-	+	-	WT	WT
HCC1569	+	-	-	WT	WT
HCC1937	-	-	-	WT	5382insC
T47D	-	+	+	mutant	WT

Key: ER: estrogen receptor, PR: progesterone receptor, +: overexpressed, -: not overexpressed, WT: wild type

Appendix 3. Tyrosine Kinase Inhibitors Acquired and Synthesized

The following table is a compilation of all the tyrosine kinase inhibitors that have been acquired and/or synthesized (14 total). These compounds have been formulated and available for the proposed cellular analyses.

kinase inhibitor	**molecular target***
erlotinib	EGFR
alkynyl gefitinib	
gefitinib	EGFR
lapatinib	EGFR, HER2
imatinib	c-Src
dasatinib	c-Abl
mastinib	c-Kit
LY294002	PI3K
U0126	MEK
SP600125	JNK
cherlerythrine chloride	PKC
ABT737	Bcl-2
NU7441	DNA-PK
AZD2281	PARP1

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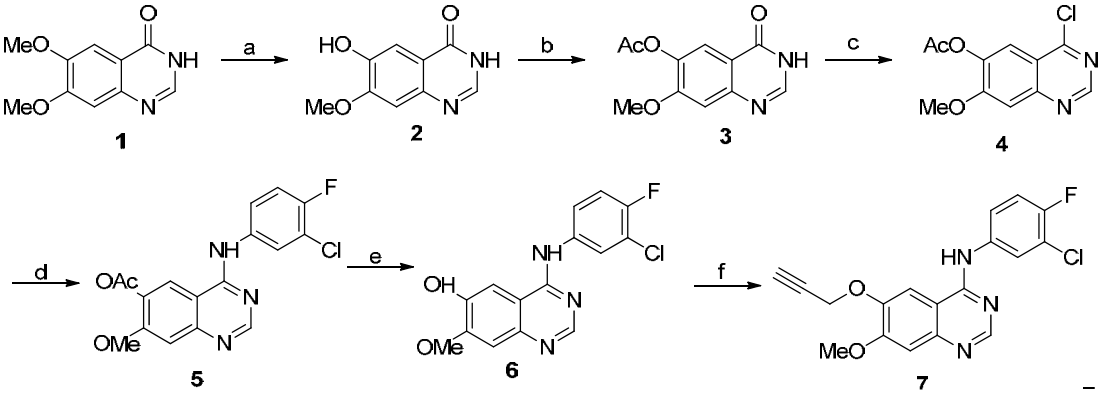
* The molecular target listed may not be the only target for the kinase inhibitor. Most kinase inhibitors act almost like pan kinase inhibitors at high concentrations.

The synthesis of alkynyl gefitinib was performed to produce a moiety in which a nuclear targeting moiety can be attached for delivery of the gefitinib moiety to the nucleus. The synthetic steps to alkynyl gefitinib, **7**, have been performed (Scheme 1). The purpose of testing this inhibitor is to observe if targeting nuclear kinases is a viable option for treatment of patients with refractory or drug resistant breast cancers.

Scheme 1. Synthesis of Alkynyl Gefitinib.



Conditions: (a) methanesulfonic acid, L-methionine, 110-120^{o}C, 6 h, 88%; (b) Ac_2O, pyridine, DMAP, 80%; (c) $POCl_3$, DEA; (d) 3-chloro-4-fluorophenylamine, isopropanol, 88% for 2 steps; (e) LiOH/MeOH-H_2O, 85%; (f) propargyl bromide, K_2CO_3, DMF, 79%;

Experimental

Unless stated otherwise, all reagents and solvents were used as received from commercial suppliers. Unless stated otherwise, all reactions were conducted in dried, anhydrous solvents. Filtration was generally performed through a pad of Celite. TLC analyses were performed on Merck aluminum-backed F254 silica gel plates. Flash

chromatography was performed using silica gel (ICN 60 Å silica gel, 32-63 μ) and samples were applied using CH_2Cl_2. NMR spectra were measured at the frequencies indicated on a 300 MHz Bruker instrument. All ^1H chemical shifts (δ) are relative to residual protic solvent ($CHCl_3$: δ 7.26, DMSO-d_6: δ 2.50, CD_3OD: δ 3.31 ppm), and all ^{13}C chemical shifts (δ) are relative to the solvent ($CDCl_3$: δ 77.23, DMSO-d_6: δ 39.52, CD_3OD: δ 49.00 ppm).

6-Hydroxy-7-methoxyquinazolin-4(3H)-one (**2**)
A mixture of 6,7-dimethoxyquinazolin-4(3H)-one (3.0 g, 14.5 mmol) and L-methionine (2.6 g, 17.2 mmol) was dissolved in methanesulfonic acid (20.5 mL) and heated to 110-120 ^oC for 6h. Over heated above 120 ^oC resulted in the production of 2'. When the reaction was complete, the mixture was poured into the crushed ice. Then NaOH (40% water solution) was added slowly (pH~7) resulting in precipitation of a white deposit. This product was collected by filtration, washed with water, cold ethanol, diethyl ether and dried in the air to afford the product (2.45 g, 88%) which was used without further purification.
^1H-NMR (300 MHz, DMSO-d_6): δ = 11.94 (s, I H), 9.80 (s, I H), 7.91 (s, I H), 7.36 (s, I H), 7.09 (s, I H), 3.90 (s, 3 H)

7-Methoxy-4-oxo-3,4-dihydroquinazolin-6-yl acetate (**3**)
A suspension of 6-hydroxy-7-methoxyquinazolin-4(*3H*)-one (2, 2.4 g, 12.5 mmol) in acetic anhydride (15 mL), pyridine (3.0 mL) and *N*,*N*-dimethyl-4-aminopyridine (15 mg) was stirred and heated to 100 -110 °C under Ar atmosphere for 4 h. The mixture was then poured into the crushed ice, and stirred vigorously for half an hour. The resulting white deposit was filtered, washed with water, cold EtOH, and Et_2O. The solid was further dried at 60 ^oC overnight to afford the product 3 (2.34 g, 80 %).
^1H-NMR (300 MHz, DMSO-d_6): δ = 12.21 (s, 1 H), 8.09 (s, 1 H), 7.75 (s, 1 H), 7.27 (s, 1 H), 3.91 (s, 3 H), 2.30 (s, 3 H)

4-Chloro-7-methoxyquinazolin-6-yl acetate (**4**)
To the solution of 7-methoxy-4-oxo-3,4-dihydroquinazolin-6-yl acetate (3, 468 mg, 2 mmol) and diisopropyl ethylamine (1.05 mL) in DCE (dichloroethane, 14 mL) was dropped in the phosphoryl chloride (0.28 mL). The whole mixture was immersed in a preheated oil bath (80 °C) and at this temperature stirred for 3 h. The reaction was monitored by TLC, the excess reagent and solvent were removed by rotary evaporation and under reduced pressure to afford the product 4, which can be used directly for next step.

4-[(3-Chloro-4-fluorophenyl)amino]-7-methoxyquinazolin-6-yl acetate (**5**)
A solution of 4-chloro-7-methoxyquinazolin-6-yl acetate 4 (prepared above, 2 mmol) and 3-chloro-4-fluorophenylamine (320 mg, 2.2 mmol) in i-PrOH (5 mL) was stirred and heated to reflux under Ar atmosphere for 5 h. Reaction progress was monitored by TLC and no starting materials were detected after this time. The reaction mixture was cooled to room temperature and the obtained precipitate was filtered through a glass funnel, with i-PrOH and Et_2O and dried under reduced pressure to provide the product 5 (635 mg, 88 % for 2 steps) as off-white powder.
^1H-NMR (300 MHz, DMSO-d_6): δ = 11.51 (s, br, 1 H), 8.94 (s, 1 H), 8.77 (s, 1 H), 8.01-8.09 (m, 1 H), 7.70-7.78 (m, 1 H), 7.48-7.55 (m, 2 H), 4.00 (s, 3 H), 2.38 (s, 3 H)

4-[(3-Chloro-4-fluorophenyl)amino]-7-methoxyquinazolin-6-ol (**6**)
The above 4-[(3-Chloro-4-fluorophenyl)amino]-7-methoxyquinazolin-6-yl acetate (5) (500 mg, 1.38 mmol) was dispersed in mixed solvent of methanol (35 mL) and H_2O (35 mL) at room temperature. LiOH (109 mg, 4.6 mmol) was added and the mixture was stirred at room temperature for 0.5 h. When the reaction was complete, monitored by TLC, the mixture was neutralized by the addition of diluted acetic acid. The resultant precipitate was collected by filtration, washed with water, cold EtOH, and Et_2O, and dried under reduced pressure to give the product 6 (373 mg, 85 %).
^1H-NMR (300 MHz, DMSO-d_6): δ = 9.79 (s, 1 H), 9.47 (s, 1 H), 8.45 (s, 1 H), 8.13-8.20 (m, 1 H), 7.70-7.85 (m, 2 H), 7.38 (t, *J* = 10.2 Hz, 1 H), 7.19 (s, 1 H), 3.96 (s, 3 H)

4-(3'-Chloro-40-fluoroanilino)-6-(prop-2-ynyloxy)-7-methoxyquinazoline (**7**)

To a solution of 6 (80 mg, 0.25 mmol) in DMF (3 ml) were added propargylbromide (60 l, 0.56 mmol) and K_2CO_3 (42mg, 0.30 mmol) and the mixture was stirred for 12 h at room temperature and monitored by TLC. The reaction was quenched with water and the mixture was extracted with ethyl acetate, dried over anhydrous Na_2SO4, and concentrated. Purification by column chromatography on silica gel with dichloromethane/methanol (100/1 – 100/2) to give 7 as a white solid (71 mg, 79%), which could be further purified by crystallization in MeOH.

^{1}H-NMR (300 MHz, CD_3OD) δ = 8.44 (s, 1 H), 7.99 (dd, *J* = 2.7, 6.6 Hz, 1 H), 7.76 (s, 1 H), 7.63–7.68 (m, 1 H), 7.24 (t, *J* = 9.0 Hz, 1 H), 7.14 (s, 1 H), 4.94(d, *J* = 2.4 Hz, 2 H), 3.99 (s, 3H), 3.04 (t, *J* = 2.4 Hz, 1 H).

Appendix 4. Cellular Fractionation and Analysis of Nuclear PCNA Isoforms

Nuclei Isolation and Fractionation (Aims 1b and 2b)
To investigate differences in chromatin-unbound and -bound nuclear fractions, a method for nuclei isolation and fractionation was developed. The method of nuclei isolation requires the use of a sucrose gradient and Dounce homogenization. Fractionation of nuclei is performed using differences in detergent properties. A non-ionic detergent (Triton) was effective at removing any non- or loosely-bound chromatin associated proteins, whereas, a stronger ionic detergent (SDS) was capable of removing proteins that are chromatin-bound. Both Aims 1b and 2b both require the use of nuclear fractionation to test the basic hypothesis that PCNA phosphorylation regulates PCNA stability onto chromatin. Enhanced stability of PCNA translates to an increase in PCNA function as a cancer-specific regulator of DNA repair and checkpoint. Demonstration of nuclear fractionation will be integrated into key experiments proposed to answer the specific research questions.

Development and Optimization of a Protein Isoform Profiling System (Aim 2b),
To identify changes of PCNA isoforms, a protein isoform profiling system has been developed. This platform is capable of fractionating and enriching for PCNA isoforms using free flow electrophoresis (FFE). FFE is a high-throughput technique that fractionates cellular lysate based on isoelectric point. Each of the 96 fractions was analyzed using a reverse-phase immunological assay. Detection of the reverse-phase immunological assay has been focused on fluorescence but will be shifted to SERRS as soon as all steps are further optimized.

Method development focused on PCNA isoform enrichment by optimizing the separation gradient. The final method utilizes a denatured, pH 3-10 pH gradient. This gradient enhances stability and allows for better resolution of PCNA isoforms (<0.1 pH unit between fractions in linear pH range). In addition, various additives were added to the separation buffers to stabilize the gradient from changes due to sample application.

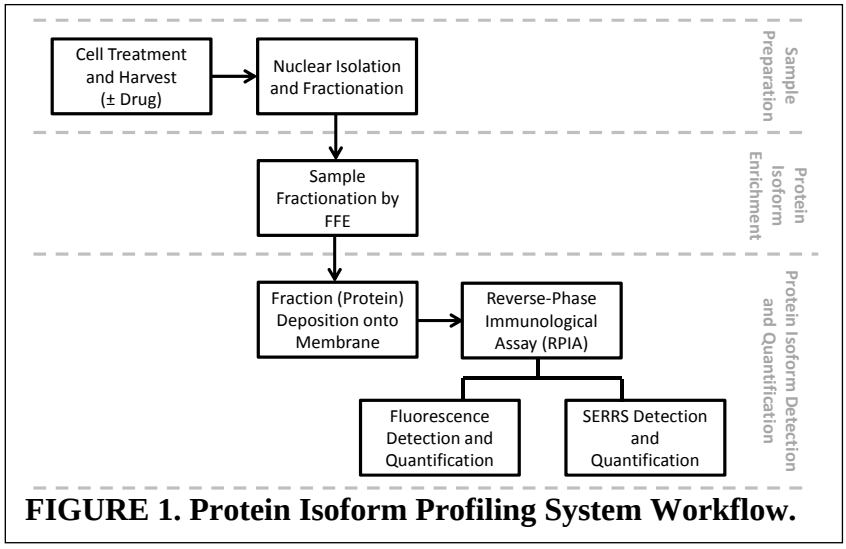


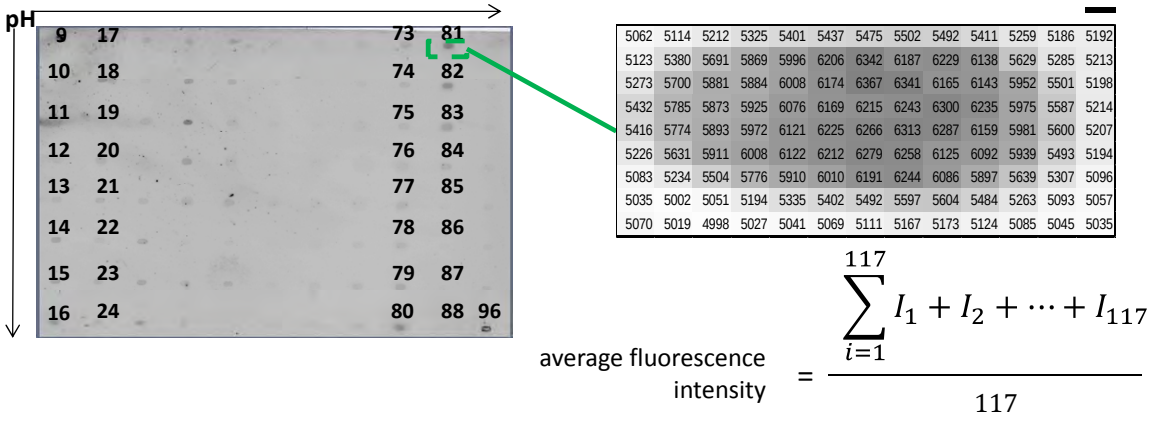
FIGURE 1. Protein Isoform Profiling System Workflow.

The major issue with the current separation gradient is the potential for protein precipitation at high concentrations of protein. When sample concentrations greater than 5 mg/mL were loaded into the separation chamber, the protein visually precipitated under denatured conditions causing shifts in the pH gradient. This resulted in poor resolution of the PCNA isoforms.

In addition, fractionating samples by FFE dilutes out the sample to the point where analysis becomes very difficult. A significant amount of time was devoted to enhancing sample detection by utilizing a variety of sample deposition and detection strategies. The best strategy currently relies on concentrating the protein through the preparation of a dot blot followed by performing a reverse-phase immunological assay (RPIA).

Detection strategies for the RPIA have been focused on use of a fluorescence readout. The fluorescence readout has allowed for faster turnaround during the isoform optimization process. More in-depth analysis was performed to look at shifts in PCNA isoform populations between runs. PCNA isoform populations were determined based on pH gradient value, and an enzyme-linked immunosorbent assay (ELISA) was developed to validate the presence of PCNA in these fractions. The ELISA assay has also been adapted to investigate the phosphorylation status of PCNA. Figure 2 shows a separation of PCNA isoform separation of MCF7 breast cancer cells using fluorescence detection.

(a)



$$\text{average fluorescence intensity} = \frac{\sum_{i=1}^{117} I_1 + I_2 + \cdots + I_{117}}{117}$$

(b)

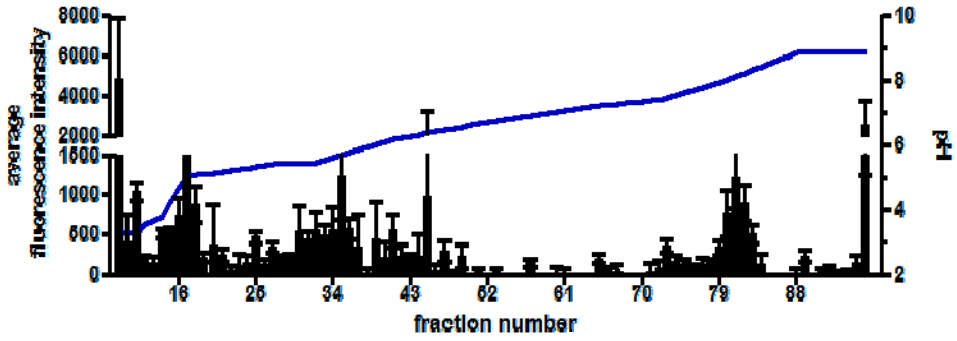
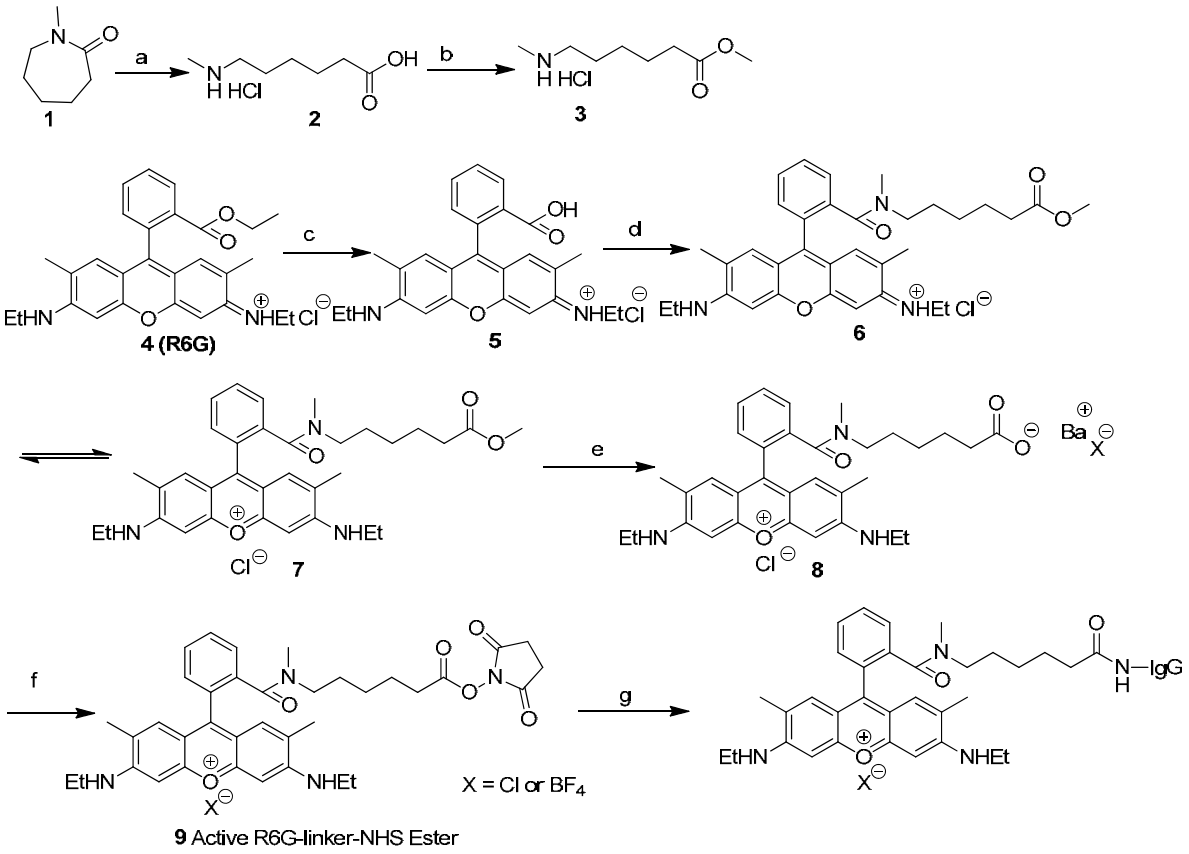


FIGURE 2. PCNA Isoform Separation as Determined by Reverse-Phase Immunological Assay. Chromatin-bound MCF7 breast cancer cell nuclear lysate was fractionated by free flow electrophoresis and the fractions were dot blotted onto a piece of PVDF membrane. A reverse-phase immunological assay was performed using a PC10 monoclonal total PCNA antibody and a fluorescently-labeled secondary antibody reporter. **(a)** Membrane was imaged using a Typhoon Trio+ image scanner using an excitation of 488nm and an emission of 526nm. Individual pixel intensities from each spot of protein deposition were averaged. **(b)** The average intensity of each spot deposition versus fraction number and pH. These demonstrate four different populations of PCNA isoforms.

The ultimate goal of this platform is to be able to quantify the levels of the various PCNA isoforms using surface-enhanced Resonance Raman spectroscopy (SERRS). Our laboratory has thoroughly characterized the properties of isotopically-labeled rhodamine-6G (R6G) fluorescent dyes for multiplex SERRS detection. The technology developed was transferred to these blots to accurately quantify the presence of various modifications on PCNA. To achieve this task, antibodies were labeled with the d_0 and d_4 encoded rhodamine-6G. Process optimization was performed for more efficient synthesis of these fluorescent dyes and antibody labeling. Scheme 1 shows the improved synthesis of d_0 and d_4-R6G for antibody labeling. The labeling efficiencies of each of these dyes were determined to be equivalent (Table 1).

A PCNA probed blot with d_0-R6G-labeled PC10 monoclonal PCNA antibody was silver and gold stained using an in-house method and Raman signals due to the fluorophore were collected. Figure 3 shows the Raman spectra collected from various fractions on PVDF membrane. This method has been shown to work well with the optimized isoform separation platform. However, further optimization is still needed to determine optimal protein deposition onto PVDF.

Scheme 1. Synthesis of R6G-Labeling Reagent

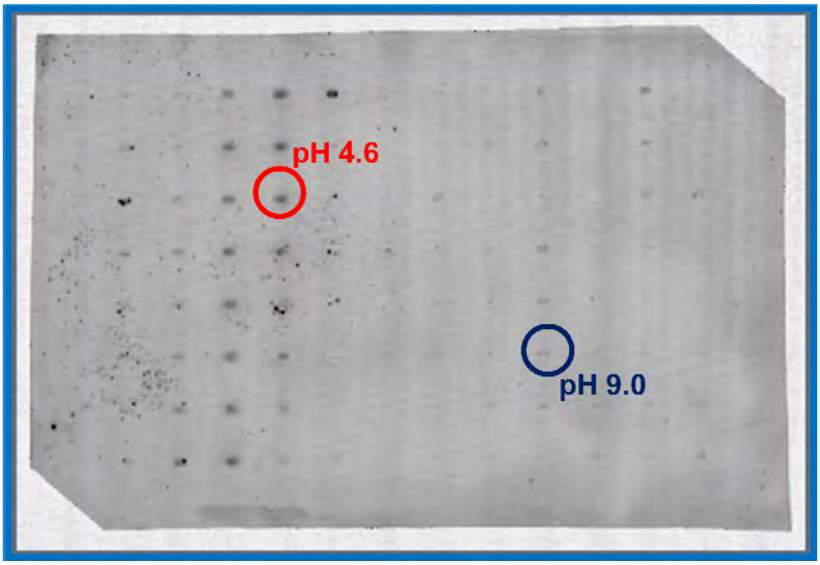


Conditions:
a): HCl (conc. aq.), reflux 5 d; b): HCl (g), MeOH, rt, overnight, quantitative for 2 steps; c): NaOH (aq. 1N), reflux 8 hr, quantitative; d): **3** (1.2 eq.), HBTU (1.2 eq.),DIPEA (4.5 eq.), DMF, rt, 6 - 8 hr 92 %; e): $Ba(OH)_2 \cdot H_2O$ (1.2 eq.), MeOH/H_2O = 2/1, rt, 18 hr; f): TSTU (1.5 eq.), ACN, rt, 5~6 hr, quantitative; g): IgG (1:100 stoichometric ratio), $NaCO_3$, pH 8.0, rt, dark, 20 mins.

Table 1. Labeling Efficiencies of d_0 and d_4-R6G Labeled IgG

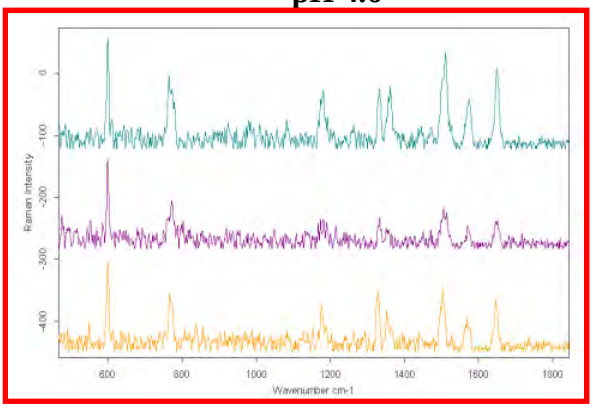
sample	A_{280}	A_{530}	Labeling Efficiency $(A_{530}/A_{280})\times 100\%$	
d_0-R6G-IgG 1:100, replicate 1	0.3436	0.2592	75.4%	
d_0-R6G-IgG 1:100, replicate 2	0.3512	0.2620	74.6%	
d_0-R6G-IgG 1:100, replicate 3	0.3424	0.2484	72.5%	74.2 ± 1.5%
d_4-R6G-IgG 1:100, replicate 1	0.2304	0.1683	73.0%	
d_4-R6G-IgG 1:100, replicate 2	0.2705	0.1810	66.9%	
d_4-R6G-IgG 1:100, replicate 3	0.2676	0.1837	68.6%	69.5 ± 3.2%

(a)



(b)

pH 4.6



pH 9.0

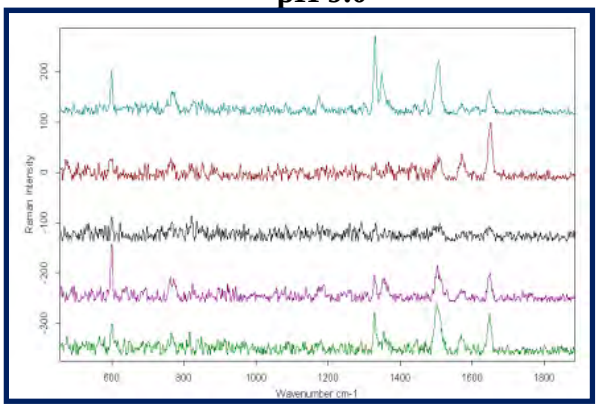


Figure 3. Surface Enhanced Resonance Raman Detection (SERRS) of PCNA Isoforms from Free Flow Electrophoresis Fractions. Nuclear chromatin-bound MCF-7 protein lysate was separated by free flow electrophoresis over a pH gradient of 3-10. A dot blot of each fraction was performed on PVDF membrane and probed for presence of total PCNA with PC10 monoclonal PCNA antibody followed by *d6*-R6G-labeled anti-mouse IgG. SERRS detection was performed on each of the spots to indicate presence of PCNA. Dot blot shows protein levels 50-fold greater amount than what was used to generate the different SERRS spectra.